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Published in:
Febs Journal

DOI:
10.1111/j.1742-4658.2007.05767.x

Publication date:
2007

the FEBS Journal

Discovery of a eugenol oxidase from *Rhodococcus* sp. strain RHA1

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Keywords
covalent flavinylation; eugenol; flavin; oxidase; *Rhodococcus*

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(Received 8 January 2007, revised 21 February 2007, accepted 2 March 2007)

doi:10.1111/j.1742-4658.2007.05767.x

A gene encoding a eugenol oxidase was identified in the genome from *Rhodococcus* sp. strain RHA1. The bacterial FAD-containing oxidase shares 45% amino acid sequence identity with vanillyl alcohol oxidase from the fungus *Penicillium simplicissimum*. Eugenol oxidase could be expressed at high levels in *Escherichia coli*, which allowed purification of 160 mg of eugenol oxidase from 1 L of culture. Gel permeation experiments and macromolecular MS revealed that the enzyme forms homodimers. Eugenol oxidase is partly expressed in the apo form, but can be fully flavinylated by the addition of FAD. Cofactor incorporation involves the formation of a covalent protein–FAD linkage, which is formed autocatalytically. Modeling using the vanillyl alcohol oxidase structure indicates that the FAD cofactor is tethered to His390 in eugenol oxidase. The model also provides a structural explanation for the observation that eugenol oxidase is dimeric whereas vanillyl alcohol oxidase is octameric. The bacterial oxidase efficiently oxidizes eugenol into coniferyl alcohol ($K_M = 1.0$ μM, $k_{cat} = 3.1$ s^{-1}). Vanillyl alcohol and 5-indanol are also readily accepted as substrates, whereas other phenolic compounds (vanillylamine, 4-ethylguaiacol) are converted with relatively poor catalytic efficiencies. The catalytic efficiencies with the identified substrates are strikingly different when compared with vanillyl alcohol oxidase. The ability to efficiently convert eugenol may facilitate biotechnological valorization of this natural aromatic compound.

The flavoenzyme vanillyl alcohol oxidase (VAO, EC 1.1.3.38) from *Penicillium simplicissimum* is active on a range of phenolic compounds [1,2]. It contains a covalently linked FAD cofactor, and the holoprotein forms stable octamers. VAO was the first histidyl-FAD-containing flavoprotein for which the crystal structure was determined [3], and serves as a prototype for a specific flavoprotein family [4]. Mutagenesis studies have shown that the covalent flavin–protein bond is crucial for efficient catalysis, and that covalent flavinylation of the apoprotein proceeds via an autocatalytic event [5,6]. As well as oxidizing alcohols, the fungal enzyme is also able to perform amine oxidations, enantioselective hydroxylations, and oxidative ether-cleavage reactions [7,8]. Several substrates can serve as vanillin precursors (e.g. vanillyl alcohol, vanillyl amine and creosol) [9,10]. Recently, VAO has been used in metabolic engineering experiments with the aim of creating a bacterial whole cell biocatalyst that is able to form vanillin from eugenol [11,12]. However, VAO is poorly expressed in bacteria, resulting in a relatively low intracellular VAO activity [12] and low yields of

Abbreviations
EUGO, eugenol oxidase; PCMH, *p*-cresol methylhydroxylase (EC 1.17.99.1); VAO, vanillyl alcohol oxidase (EC 1.1.3.38).

purified VAO when *Escherichia coli* is used as the expression host [13].

In a quest for a bacterial VAO, we have searched the sequenced bacterial genomes for VAO homologs. Such a search is complicated by the fact that bacterial hydroxylases, *p*-cresol methylhydroxylase (PCMH) [14] and eugenol hydroxylase [15,16], have been reported that show sequence identity with VAO. PCMH and eugenol hydroxylase display similar substrate specificities when compared with VAO [16–18]. For VAO and PCMH, several crystal structures have been elucidated showing that the respective active sites are remarkably conserved [3,18]. This is in line with the overlapping substrate specificities. However, a major difference between VAO and the bacterial hydroxylases is the ability of VAO to use molecular oxygen as electron acceptor. Instead, the bacterial hydroxylases employ cytochrome domains to relay the electrons towards azurin as electron acceptor. Another difference between VAO and the bacterial hydroxylases is the mode of binding of the FAD cofactor. In VAO, FAD is covalently bound to a histidine, whereas the bacterial counterparts contain a tyrosyl-linked FAD cofactor [3,19]. It has been shown that in PCMH, the electron transfer from the reduced flavin cofactor to the cytochrome subunit is facilitated by the covalent FAD–tyrosyl linkage. For VAO, it has been demonstrated that the covalent FAD–histidyl linkage induces a relatively high redox potential, allowing the enzyme to use molecular oxygen as electron acceptor [5].

By surveying the available sequenced genomes, a number of VAO homologs can be found: 25 bacterial and fungal homologs with sequence identity of $> 30\%$. A putative VAO from *Rhodococcus* sp. strain RHA1 was found to display sequence identity with VAO (45%) (40% with PCMH). Sequence alignment with its characterized homologs revealed that it contains a histidine residue (His390) at the equivalent position of the FAD-binding histidine in VAO (Fig. 1). This suggested that this enzyme might represent a bacterial VAO. In this article, we describe the production, purification and characterization of this novel oxidase from *Rhodococcus* sp. strain RHA1. The bacterial oxidase was found to be most active with eugenol, and hence has been named eugenol oxidase (EUGO).

Results

Properties and spectral characterization of EUGO

EUGO can be expressed at a remarkably high level in *E. coli* TOP10 cells (Fig. 2, lane 2a). From a 1 L culture, about 160 mg of yellow-colored recombinant EUGO was purified. The purified enzyme migrated as a single band on SDS/PAGE, corresponding to a mass of about 58 kDa (Fig. 2, lane 4a). This agrees well with the predicted mass of 58 681 Da (excluding the FAD cofactor). A fluorescent band was visible when the gel was soaked in 5% acetic acid and placed under UV light. This indicates that a flavin cofactor is covalently linked to the enzyme. Unfolding and precipitation by trichloroacetic acid resulted in formation of a yellow protein aggregate, which confirms that the flavin cofactor is covalently bound to the protein.

The purified enzyme showed absorption maxima in the visible region at 365 nm and 441 nm, and shoulders at 313 nm, 394 nm, and 461 nm (Fig. 3). Upon unfolding of the enzyme in 0.5% SDS, the absorption maximum at 441 nm slightly decreased in intensity and shifted to 450 nm. If it is assumed that the molar absorption coefficient of the unfolded enzyme is comparable to that of 8α-substituted FAD [20], a value of 14.2 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ can be calculated for the molar extinction coefficient of the native enzyme. These spectral characteristics are very similar to those of VAO [1], indicating that the FAD cofactor is in a similar microenvironment and histidyl-linked. The presence of a histidyl-linked FAD cofactor agrees with the model that could be prepared of EUGO. The structural model shows that His390 is in a similar position to the FAD-linking His422 in VAO (Fig. 4).

It has been observed that most flavoprotein oxidases can form a stable covalent adduct with sulfite. However, the purified enzyme did not form such a covalent sulfite–flavin adduct, as no spectral changes occurred upon incubation with 10 mM sulfite. A similar reluctance to react with sulfite has been observed with a selected number of flavoprotein oxidases, including VAO from *P. simplicissimum* [1].

Catalytic properties of EUGO

Like VAO from *P. simplicissimum*, EUGO exhibits a wide substrate spectrum. Table 1 shows the steady-state kinetic parameters of the bacterial oxidase with all identified phenolic substrates. It is evident that eugenol is the best substrate, and therefore we have named the enzyme eugenol oxidase. Aerobic incubation of eugenol with EUGO led to full conversion into coniferyl alcohol, as judged by formation of a typical UV–visible spectrum indicative for this aromatic compound (Fig. 5). The same hydroxylation reaction with eugenol has been described for VAO and eugenol hydroxylase, which includes attack by water to form the hydroxylated product coniferyl alcohol [2,16].

Fig. 1. Multiple sequence alignment of VAO homologs. The sequences are: EUGO from *Rhodococcus* sp. strain RHA1 (gi111020271/ro03282); VAO from *P. simplicissimum* (gi3024813); hydroxylase subunit of PCMH from *Pseudomonas putida* (gi62738319); and hydroxylase subunit of eugenol hydroxylase (EUGH) from *Pseudomonas* sp. strain HR199 (gi6634499). The histidine and tyrosine residues that are covalently linked to the FAD cofactor are in bold.

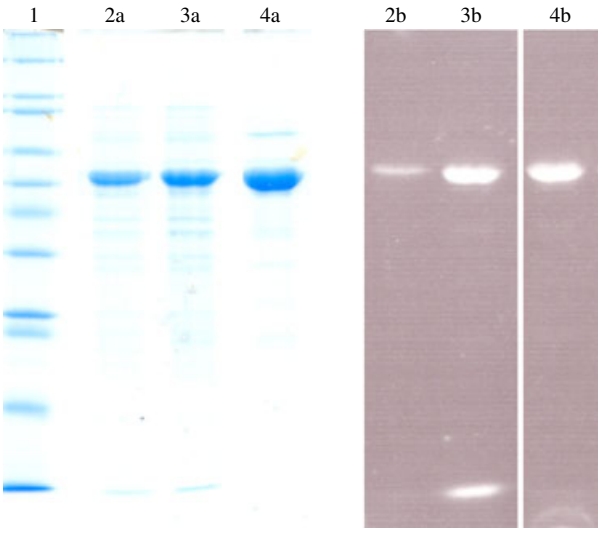


Fig. 2. Recombinant EUGO analyzed by SDS/PAGE. Lane 1: marker proteins (from top to bottom: myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; BSA, 66 kDa; glutamic dehydrogenase, 55 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14.2 kDa; aprotinin, 6.5 kDa). Lane 2a: protein-stained cell-free extract. Lane 3a: protein-stained cell-free extract that had been incubated with 200 μM FAD. Lane 4a: protein-stained purified EUGO. Lanes 2b, 3b and 4b are identical to lanes 2a, 3a and 4a, but represent flavin fluorescence.

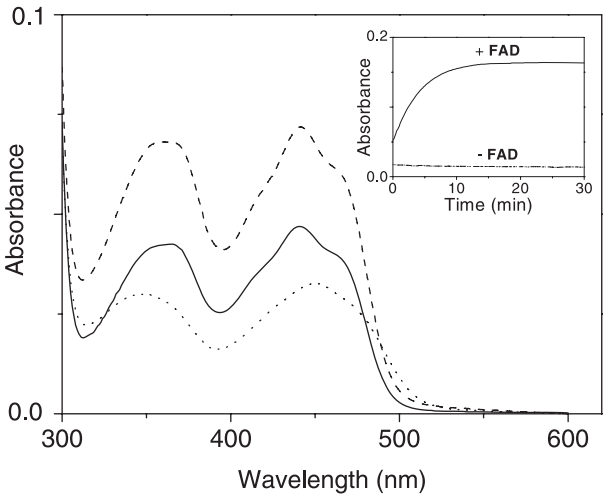


Fig. 3. Visible spectra of native EUGO (solid line), after unfolding by 0.5% SDS (dotted line) and fully flavinylated EUGO (dashed line). The figure shows the spectral changes observed upon incubation of purified EUGO with SDS and additional FAD: 6.0 μM EUGO before incubation with FAD (solid line), after incubation with 0.5% SDS (dotted line) and after 60 min of incubation with 100 μM FAD and subsequent ultrafiltration (dashed line). The inset shows formation of hydrogen peroxide during incubation of 18 μM EUGO with 100 μM FAD (solid line) or without FAD (dotted line).

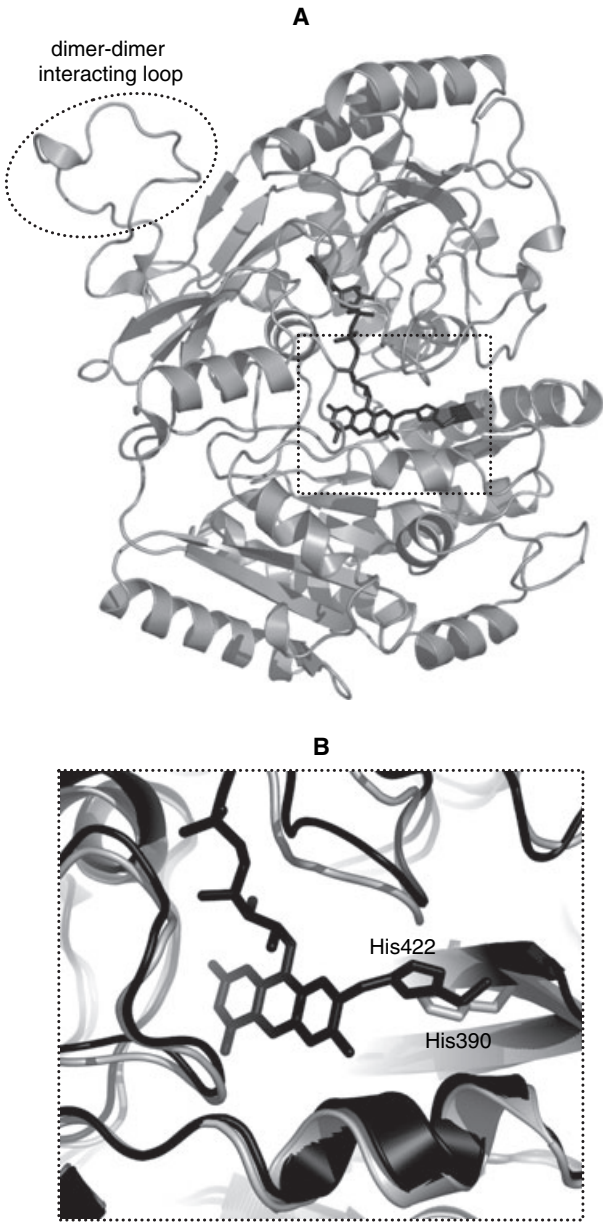
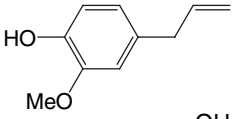
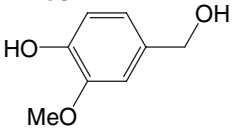
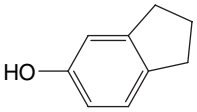
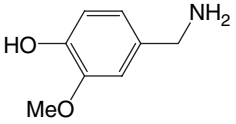
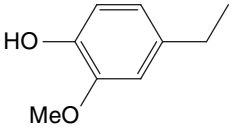
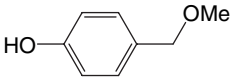


Fig. 4. (A) Crystal structure of VAO in which the histidyl-bound FAD cofactor is shown in sticks [3]. The dimer–dimer interacting loop, missing in EUGO, is indicated. (B) Superposition of the VAO structure (black) and the modeled apo-EUGO structure (gray). His422 of VAO, linking the FAD cofactor, aligns with His390 of EUGO.

Although EUGO accepts a similar range of substrates as VAO, there are some marked differences. The catalytic efficiencies (k_{cat}/K_M) for vanillyl alcohol and 5-indanol are higher than those of VAO, whereas vanillylamine and alkylphenols are relatively poor substrates for the bacterial oxidase. The proposed physiologic substrate for VAO, 4-(methoxymethyl)-phenol, is

Table 1. Steady-state kinetic parameters for recombinant EUGO and VAO. The kinetic parameters of EUGO, as isolated, were measured at 25 °C in 50 mM potassium phosphate buffer (pH 7.5). All kinetic parameters given for VAO have been reported before [2,10,21]. ND, not determined.

		EUGO			VAO		
Substrate		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (10^3 $s^{-1}\cdot M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (10^3 $s^{-1}\cdot M^{-1}$)
Eugenol		1.0	3.1	3100	2	14	7000
Vanillyl alcohol		40	12	300	75	1.6	21
5-Indanol		23	2.4	100	77	0.5	7
Vanillylamine		76	0.26	3.4	240	1.3	5.4
4-Ethylguaiacol		2.1	0.026	12	ND	ND	ND
4-(Methoxymethyl)phenol		2.3	0.004	2	58	3.1	53

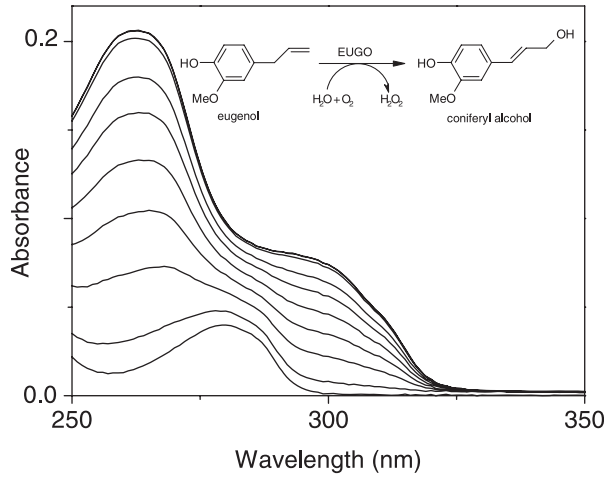


Fig. 5. Absorption spectra during conversion of eugenol by EUGO. The reaction mixture contained 0.010 mM eugenol in 1.0 mL of 50 mM potassium phosphate (pH 7.5). Spectra (from the bottom to top) were recorded at 0, 2, 4, 6, 8, 10, 12, 14 and 16 min after the addition of 0.01 nmol of EUGO.

hardly accepted by EUGO. By measuring oxygen consumption, it was found that EUGO is able to oxidize substrates by using molecular oxygen. Addition of 50 U of catalase after complete conversion of 0.2 mM eugenol resulted in the formation of 0.1 mM molecular oxygen. This shows that oxygen consumption is accompanied by hydrogen peroxide formation, which confirms that EUGO is a true oxidase.

With vanillyl alcohol as substrate, the pH optimum for enzyme activity was determined. The isolated enzyme has a broad pH optimum for activity, with more than 90% of its maximum activity being between pH 9 and 10. The enzyme, as isolated, is reasonably stable, as no inactivation occurred after incubation of the oxidase (3.4 μM EUGO in 20 mM Tris/HCl, pH 7.5) for 90 min at 45 °C. With incubation at 60 °C, the enzyme showed an activity half-life of 30 min. Addition of a three-fold excess of FAD to the incubation mixture resulted in a 1.5-fold longer half-life of activity (45 min). This indicates that FAD binding is beneficial for enzyme stability.

Structural properties of EUGO

Macromolecular MS was used to determine the exact molecular mass of EUGO. For this, purified enzyme was dissolved in a denaturing solution (50% acetonitrile and 0.2% formic acid), and analyzed in a concentration of 1 μM by nanoflow ESI MS. Under these acidic conditions, EUGO takes up a high number of charges, from which an accurate mass can be determined. Four protein species were observed in different ratios: a, 58 549 ± 5 Da; b, 58 681 ± 2 Da; c, 59 334 ± 2 Da; d, 59 465 ± 2 Da (Fig. 6A). The measured mass of species b is in very good agreement with the expected mass on the basis of the EUGO primary sequence (58 681 Da). Therefore, species b represents apo-EUGO, whereas species d represents EUGO covalently bound to an FAD cofactor (+ 785 Da). Species c is the flavinylated form of EUGO without the N-terminal methionine, whereas species a is the corresponding apo form. The mass spectrum suggests that 37 ± 2% EUGO was present in the apo form and 63 ± 2% in the holo form. The oxidase did not contain any noncovalently bound FAD, as under denaturing conditions no free FAD was detected in the mass spectrum.

Using a Superdex-200 column, the apparent molecular mass of native EUGO was estimated to be 111 kDa. No other oligomeric forms were observed. Because each subunit is 59 kDa, the gel permeation experiments indicate that the enzyme is mainly homodimeric in solution. In order to analyze the EUGO dimer molecules in more detail, mass spectra of the protein were recorded under native conditions (50 mM ammonium acetate, pH 6.8), as described for VAO [22]. When EUGO monomer was sprayed at a concentration of 1 μM, the mass spectrum showed six different species in different ratios (Fig. 6C). All observed species represent dimeric forms of EUGO: e, 117 908 Da; f, 118 053 Da; g, 118 176 Da; h, 118 706 Da; i, 118 833 Da; and j, 118 958 Da. The determined molecular masses for all the species were always higher (between 23 and 37 Da) than the predicted masses based on the primary sequence, which can be explained by the presence of one or two water molecules in the protein oligomer. The mass spectrum showed that 53 ± 6% of the dimeric protein molecules (species e, f and g) contain one FAD covalently bound, and 47 ± 6% (species h, i and j) contain two FADs covalently bound. Thus, no dimer without any FAD molecule was observed. Species e and h correspond to dimeric enzyme in which the N-terminal methionine has been removed in both monomers. Species g and j match the mass of dimeric EUGO, in which both monomers contain the N-terminal methionine. Species f and i correspond to dimeric EUGO in which one monomer contains the N-terminal methionine and the other does not.

Flavinylation of EUGO

The MS experiments indicated that EUGO, as isolated, was not fully saturated with its FAD cofactor. To determine whether the copurified apo form could be reconstituted, the enzyme was mixed with FAD and the mixture was monitored in real time by MS. The mass spectrum obtained after 10 min of incubation (Fig. 6D) revealed the presence of only three species with two FAD molecules covalently bound. These species, h, i and j, correspond to EUGO dimer molecules without an N-terminal methionine, one N-terminal methionine and two N-terminal methionine residues, respectively. This was also confirmed by MS under denaturing conditions after incubation of the isolated oxidase with FAD for 10 min (Fig. 6B). During the incubation, the apo form (species a and b) completely transformed to the holo form, with one FAD covalently bound (species c and d).

Successful incorporation of the FAD cofactor was also shown by incubation of the enzyme for 1 h with 200 μM FAD. After removal of the excess FAD with an Amicon YM-10 filter, a significant increase (56%) in enzyme activity was measured. This is in agreement with the observation that the ratio of protein/flavin absorbance increased after incubation with excess FAD. The A_{280}/A_{441} ratio of EUGO, as purified, was 12.5, whereas incubation with FAD resulted in a ratio of 8.3 (Fig. 3). The spectral shapes of enzymes partly and fully in the holo form were identical. This indicates that the microenvironment around the FAD cofactor in the *in vitro* reconstituted enzyme is similar to that in the native holo-EUGO. SDS/PAGE analysis of FAD-incubated EUGO resulted in an increase in flavin fluorescence (Fig. 2, lane 3). This shows that the cofactor incorporation leads to covalent attachment of the FAD cofactor.

The successful *in vitro* cofactor incorporation shows that the covalent incorporation is an autocatalytic process. Covalent flavinylation is postulated to involve the formation of a reduced flavin intermediate [23,24]. It has been proposed that reoxidation of the reduced flavin intermediate is accomplished by using molecular oxygen as electron acceptor. As a consequence, the reoxidation should be accompanied by formation of hydrogen peroxide [25]. Hydrogen peroxide can be detected by using a horseradish peroxidase-coupled assay with 3,5-dichloro-2-hydroxybenzenesulfonic acid

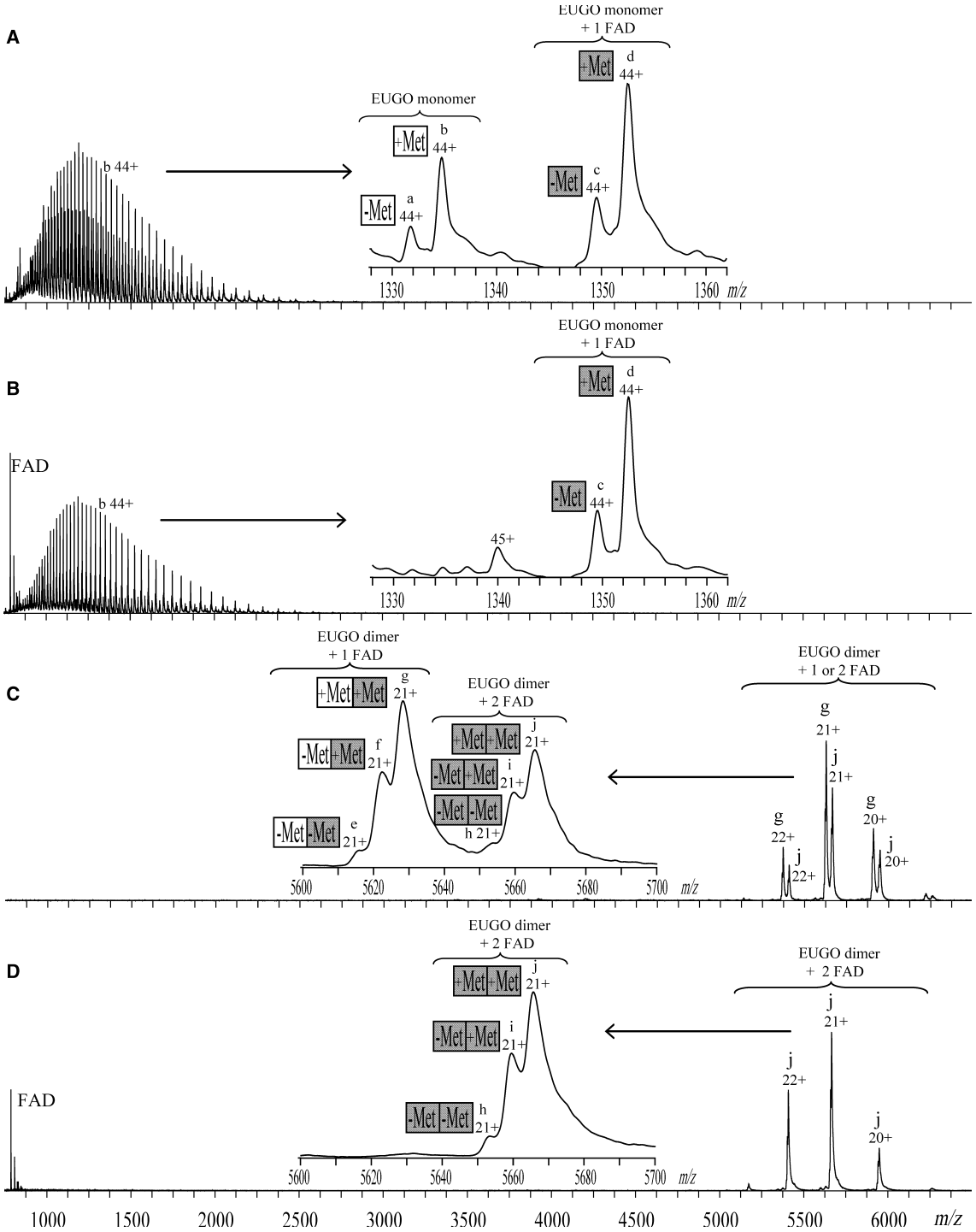


Fig. 6. (A, B) Mass spectra obtained under denaturing conditions of EUGO (A) and EUGO incubated for 10 min at room temperature with a four-fold molar excess of FAD (B). EUGO in 50 mM ammonium acetate buffer (pH 6.8) was denatured by dilution in a solution with 50% acetonitrile and 0.2% formic acid, and sprayed at a concentration of 1 μM into the mass spectrometer. a, b, c and d represent the different species of the monomeric EUGO. (C, D) Native mass spectra of EUGO sprayed from a 50 mM ammonium acetate buffer (pH 6.8) at 1 μM in the absence (C) or the presence (D) of 4 μM FAD incubated for 10 min. The charges of the different ion series are indicated. e, f, g, h, i and j correspond to the different species of the dimeric EUGO. The monomer of EUGO is presented by a white or gray square corresponding to the absence or presence, respectively, of one FAD molecule covalently bound. –Met and +Met correspond to the absence or presence of the N-terminal methionine in the monomer.

and 4-aminoantipyridine as chromogenic substrates. Oxidation of these latter two compounds leads to formation of *N*-(4-antipyryl)-3-chloro-5-sulfonate-*p*-benzoquinonemonoimine. This results in a large increase in absorbance at 515 nm ($\varepsilon_{515} = 26$ mM^{-1}·cm^{-1}), whereas FAD does not give significant interfering absorbance at this wavelength. As is shown in the inset of Fig. 3, hydrogen peroxide is formed upon adding FAD to a reaction mixture containing EUGO, horseradish peroxidase, 3,5-dichloro-2-hydroxybenzenesulfonic acid, and 4-aminophenazone. The amount of hydrogen peroxide produced (4.30 μM) was similar to the amount of apo-EUGO present in the incubation mixture (4.27 μM), as estimated on the basis of the increase in oxidase activity.

Discussion

In this study, a new bacterial oxidase was cloned and characterized: EUGO from *Rhodococcus* sp. strain RHA1. The oxidase shows sequence identity (45%) with the fungal VAO, and is also closely related in sequence to the bacterial PCMH (40% sequence identity). EUGO represents a true oxidase, as it can efficiently use molecular oxygen as electron acceptor. It shares this property with VAO, whereas PCMH is not able to utilize molecular oxygen as electron acceptor. This study has revealed that EUGO also shares another feature of VAO: it contains a histidyl-bound FAD cofactor. This is in line with the observation that VAO homologs that contain a histidyl-bound FAD often act as oxidases [4]. The substrate specificity of EUGO shows some overlap with that of VAO. The best substrate identified for EUGO is eugenol, and vanillyl alcohol and 5-indanol are also readily accepted. The latter compound is a poor substrate for VAO, whereas the proposed physiologic substrate of VAO (4-methoxymethyl)phenol [8]) is poorly converted by EUGO. This suggests that EUGO has not evolved to oxidize the same physiologic substrate as VAO, but may be involved in the degradation of 5-indanol or related aromatic compounds. The sequenced genome of *Rhodococcus* sp. strain RHA1 has revealed that this actinomycete has an extensive repertoire of enzymes acting on aromatic compounds [26]. The *in vivo* aromatic substrate for EUGO remains to be determined. Inspection of the sequence regions neighboring the *eugo* gene (ro03282) reveals that it is flanked by the genes for two putative aldehyde dehydrogenases (ro03281 and ro03284), and that for a putative aryl-alcohol dehydrogenase (ro03285). The clustering of the catabolic genes again hints at a role for EUGO in the degradation of aromatic compounds. The absence of a gene located nearby encoding a cytochrome again confirms that EUGO is not, like PCMH, a flavocytochrome.

The high level of sequence similarity with VAO allowed modeling of EUGO. Comparison of the modeled structure with the structure of VAO revealed that the active sites are remarkably conserved. All residues that have previously been shown to be involved in binding the phenolic moiety of VAO substrates are conserved [3]. Only residues that form the cavity that accommodates the *p*-alkyl side chain are less well conserved. This may explain the observed differences in substrate specificity. A striking structural difference between VAO and EUGO is that EUGO lacks the loop formed by residues 218–235 in VAO (Figs 1 and 4). In VAO, this loop is involved in dimer–dimer interactions resulting in the formation of holo-octamers. This explains why EUGO is a dimeric protein not able to stabilize octamers. It is also in line with the observation that PCMH and eugenol hydroxylase are heterotetramers consisting of a dimer of flavoprotein subunits flanked by two cytochromes. These hydroxylases also lack the dimer–dimer interacting loop that promotes octamerization in VAO (Fig. 1).

Macromolecular MS and cofactor incorporation experiments revealed that recombinant EUGO is, to a large extent, expressed in its apo form. As the enzyme is highly overexpressed in *E. coli*, the presence of apo-EUGO can be explained by a lack of intracellular oxygen or the fact that the *E. coli* cells cannot produce enough FAD for complete flavinylation of the dimeric enzyme. From the MS experiments, it can be concluded that about half of the purified dimeric recombinant EUGO contains only one FAD cofactor. Remarkably, no apo dimeric enzyme was observed, which suggests that at least one FAD is necessary to stabilize the dimeric form of EUGO. The partially apo form of EUGO became fully flavinylated *in vitro* by the addition of FAD. The cofactor incorporation resulted in formation of holo dimeric EUGO, in which all FAD is covalently bound. Covalent flavinylation was accompanied by an increase in oxidase activity and formation of hydrogen peroxide. This confirms a mechanism of autocatalytic covalent flavinylation in which a reduced histidyl–flavin intermediate is produced. Reoxidation of this intermediate is accomplished by using molecular oxygen as electron acceptor, resulting in the formation of hydrogen peroxide. Such an autocatalytic oxidative mechanism of FAD coupling was recently also demonstrated for sarcosine oxidase [25].

Flavoprotein oxidases are valuable biocatalysts for synthetic applications, with broad substrate specificity

[27]. Because of their ability to utilize molecular oxygen as a mild oxidant, EUGO and VAO appear to be more attractive for biocatalytic purposes when compared with the bacterial hydroxylases PCMH and eugenol hydroxylase, which need a proteinous electron acceptor [28]. It has been shown that the expression level of recombinant VAO in *E. coli* is poor when the original fungal gene is used. Gene optimization has been reported to alleviate this problem [29]. This study shows that EUGO can be produced in large quantities in *E. coli* and can be purified with ease. Therefore, it represents a good alternative biocatalyst for the enzymatic synthesis of vanillin and related phenolic compounds.

Experimental procedures

Chemicals

Restriction enzymes, DNA polymerase and T4 DNA ligase were obtained from Roche (Basel, Switzerland). Eugenol (4-allyl-2-methoxymethylphenol), creosol, 4-(methoxymethyl) phenol, 4-ethylguaiacol (4-ethyl-2-methoxyphenol), and 5-indanol were products of Sigma-Aldrich (St Louis, MO, USA). Vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), vanillylamine hydrochloride (4-hydroxy-3-methoxybenzylamine hydrochloride), epinephrine [l-3,4-dihydroxy-α-(methylaminomethyl)benzyl alcohol] and L-(+) arabinose were obtained from Acros (Geel, Belgium). DNA samples were purified using the QIAquick gel and purification kit from Qiagen (Valencia, CA, USA). *E. coli* TOP10-competent cells and the pBAD/myc-HisA vector were purchased from Invitrogen (Carlsbad, CA, USA).

Expression and purification of recombinant EUGO

DNA from *Rhodococcus* sp. strain RHA1 was a kind gift from R v.d. Geize (University of Groningen, The Netherlands). The *eugo* gene (gi111020271) was amplified using genomic DNA from *Rhodococcus* sp. strain RHA1 and the following primers: forward, 5′-CACCATATGACG CGAACCCTTCCCCCA-3′ (*Nde*I site is underlined); and reverse, 5′-CACAAGCTTCAGAGGTTTTGGCCACGG-3′ (*Hin*dIII site is underlined). After amplification, the DNA was digested with *Nde*I and *Hin*dIII, purified from agarose gel, and ligated between the same restriction sites in pBADNk, a pBAD/myc-HisA-derived expression vector in which an original *Nde*I site is removed and the *Nco*I site is replaced by an *Nde*I site. The plasmid thus obtained was named pEUGOA, and transformed into *E. coli* TOP10 cells. For expression, the *E. coli* TOP10 cells harboring pEUGOA were grown in Terrific Broth medium supplemented with 50 $\mu g \cdot mL^{-1}$ ampicillin and 0.02% (w/v) arabinose at 30 °C. Cells from 1 L of culture were harvested by centrifugation at 4000 ***g***, (Beckman J2-21 M/E centrifuge with a JA-10 rotor), and resuspended in 25 mL of potassium phosphate buffer, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 0.5 mM $MgSO_4$ (pH 7.0). Cells were disrupted by sonication at 20 kHz for 20 min at 4 °C. Following centrifugation at 23 000 ***g***, (Beckman J2-21 M/E centrifuge with a JA-17 rotor) to remove cellular debris, the supernatant was applied to a Q-Sepharose column pre-equilibrated in the same buffer. The enzyme was eluted with a linear gradient from 0 to 1.0 M KCl in the same buffer. Fractions were assayed for VAO activity, pooled, desalted, and concentrated in an Amicon ultrafiltration unit (Millipore, Billerica, MA, USA) equipped with a YM-30 membrane.

Analytical methods

Enzyme activity was routinely assayed by following the changes in absorption. Activity with vanillyl alcohol and vanillylamine was determined by measuring the formation of vanillin at 340 nm ($\varepsilon = 14.0\ mM^{-1} \cdot cm^{-1}$ at pH 7.5). The formation of coniferyl alcohol from eugenol was measured at 296 nm ($\varepsilon = 6.8\ mM^{-1} \cdot cm^{-1}$ at pH 7.5). Activity against 4-ethylguaiacol and 5-indanol was determined by measuring the increase of absorption at 255 nm ($\varepsilon = 50\ mM^{-1} \cdot cm^{-1}$ at pH 7.5) and 300 nm ($\varepsilon = 11.5\ mM^{-1} \cdot cm^{-1}$ at pH 7.5), respectively. When the pH optimum of the enzyme was measured using vanillyl alcohol as substrate, the activity was corrected for the pH dependence of the molar extinction coefficient of vanillin. Oxygen consumption and formation was monitored in a 1 mL stainless-steel stirred vessel using an optical oxygen sensor 'Mops-1' (Prosense, Hannover, Germany). With this method, hydrogen peroxide concentrations up to 25 μM could be measured.

The cofactor incorporation reactions were conducted at 25 °C in 50 mM potassium phosphate buffer (pH 7.5) containing 1.3 μM EUGO, 20 U of horseradish peroxidase, 0.1 mM 4-aminoantipyridine, and 1.0 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid. Flavinylation of the isolated enzyme was initiated by the addition of 200 μM FAD. Hydrogen peroxide formation was monitored at 515 nm ($\varepsilon_{515} = 26\ mM^{-1} \cdot cm^{-1}$) [30].

Analytical size-exclusion chromatography was performed with a Superdex 200 HR 10/30 column (Amersham Biosciences, Piscataway, NJ, USA), using 50 mM potassium phosphate buffer (pH 7.5). Aliquots of 100 μL were loaded on the column and eluted at a flow rate of $0.4\ mL \cdot min^{-1}$. Apparent molecular masses were determined using a calibration curve made with standards from the Bio-Rad molecular marker kit (Hercules, CA, USA): thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa).

For nanoflow ESI MS experiments, enzyme samples were prepared in aqueous 50 mM ammonium acetate buffer

(pH 6.8). The flavinylation reaction was initiated by addition of a four-fold molar excess of FAD. For measurements under denaturing conditions, the protein was diluted in a solution containing 50% acetonitrile and 0.2% formic acid. EUGO samples (1 μM) were introduced into an LC-T nanoflow ESI orthogonal TOF mass spectrometer (Micromass, Manchester, UK), operating in positive ion mode, using gold-coated needles. The needles were made from borosilicate glass capillaries (Kwik-Fil; World Precision Instruments, Sarasota, FL) on a P-97 puller (Sutter Instruments, Novato, CA), and coated with a thin gold layer by using an Edwards Scancoat (Edwards Laboratories, Milpitas, CA) six Pirani 501 sputter coater. All the mass spectra were calibrated using cesium iodide (25 $mg \cdot mL^{-1}$) in water. Source pressure conditions and electrospray voltages were optimized for transmission of EUGO oligomers [31,32]. The needle and sample cone voltages were 1300 V and 160 V, respectively. The pressure in the interface region was adjusted to 8 mbar by reducing the pumping capacity of the rotary pump by closing the speedivalve.

The structural model of EUGO was prepared using the CPHMODELS 2.0 Server (http://www.cbs.dtu.dk/services/CPHmodels). The model was built using the crystal structure of the VAO mutant H61T (Protein Data Bank 1E8F), and pictures were prepared using PYMOL software (pymol.sourceforge.net).

Acknowledgements

This research was supported by the Dutch Technology Foundation STW, the applied science division of NOW, and the Technology Program of the Ministry of Economic Affairs.

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